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PRINCIPAL INVESTIGATOR: William C. Hines
Jeffrey K. Griffith, Ph.D.

CONTRACTING ORGANIZATION: University of New Mexico Health Science Center
Albuquerque, New Mexico 87131

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6. AUTHOR(S)
William C. Hines
Jeffrey K. Griffith, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
University of New Mexico Health Science Center
Albuquerque, New Mexico 87131

E-Mail: curthines@aol.com

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13. ABSTRACT (Maximum 200 Words)

The aims of this study are to identify the types and properties of telomerase producing cells within breast tumors, and further, to isolate these cells from breast tumors so that their biochemical and functional properties may be characterized. Through examining the role of telomerase in cancer, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, epidemiology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all tasks; as outlined in the Statement of Work, are on schedule. The research is in progress.

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Real time RT-PCR, telomerase, hTERT, in-situ hybridization (ISH), immunohistochemistry (IHC), fluorescence activated cell sorting (FACC)

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I. INTRODUCTION

The aims of this study are to identify the types and properties of telomerase producing cells within breast tumors, and further, to isolate these cells from breast tumors so that their biochemical and functional properties may be characterized. Through examining the role of telomerase in cancer, this project also fosters the education of the candidate through the interaction with several experts in breast cancer pathology, epidemiology, biostatistics, and clinical and basic research. The experiments involved require the interaction with professionals from several different fields of the biomedical sciences and the mastery of several challenging laboratory techniques. To date, all tasks; as outlined in the Statement of Work, are on schedule. The research is in progress.

Hypothesis and Rationale

To understand the effects of hTERT on breast cancer cell immortalization and tumorigenesis, it is necessary to study telomerase expression at the level of the individual cell. The preliminary experiments and the evidence in the current literature suggest that: 1) the levels of hTERT expression vary within the cellular subpopulations of a tumor and/or 2) the fraction of cells expressing telomerase varies between tumors. Based on these considerations, ***I hypothesize that breast cancer cells with the highest expression of hTERT will have the most aggressive phenotype.*** I will evaluate this hypothesis by testing the predictions in two specific aims.

- **Specific Aim #1**
  To identify the types, numbers, and properties of breast tumor cells expressing hTERT, so as to define the variability in cellular expression of hTERT in human breast tumors.

- **Specific Aim #2**
  To characterize the biochemical and functional properties of breast tumor cells expressing elevated hTERT, so as to determine if these cells have the more aggressive phenotype that is correlated with a poor clinical outcome.

II. Key Research Accomplishments

IIa. RESEARCH ACCOMPLISHMENTS

- Preliminary results indicate that Antibodies to Cytokeratin, Epithelial membrane antigen, Vimentin, alpha actin, and common acute lymphocytic leukemia antigen (CALLA) will be sufficient when used to characterize Epithelial cells, fibroblasts, and myoepithelial cells derived from breast tumors. (several of these are contained within Appendix C)

- hTERT FISH assay has revealed hTERT expression by epithelial cells within tumors, with intensities that correspond to the hTERT mRNA levels as measured by real-time RT-PCR. (Appendix B, C)

- There exists an association between levels of hTERT and cmyc mRNA within breast tumors, as measured by real-time RT-PCR (Appendix F,I).

- The levels of hTERT mRNA are not associated with mRNA levels of the telomere binding proteins TRF-1 and TRF-2 within breast tumors (Appendix G).

IIb. TRAINING/EDUCATIONAL ACCOMPLISHMENTS

Since the activation of this award the PhD candidate has had the opportunity to work and interact with oncologists, pathologists and other Ph.D. scientists who specialize in breast cancer. This has principally through tumor board meetings, journal clubs, special seminars and direct interaction within the laboratory. He has been trained by experts that oversee the Microscopy and Flow Cytometry core facilities at the UNM Health Sciences Center. The Ph.D. candidate's research is overseen by his research committee, a body comprised of three Ph.D. scientists with interests in breast cancer, and one M.D. that specializes in breast cancer pathology.

On an educational level, the candidate continues to instruct for three upper-level courses: 1) Biochemical Laboratory Methods, 2) Intensive Biochemistry I, and 3) Intensive Biochemistry II: Intermediary metabolism. The candidate has aspirations of continuing his research and remaining in academia and felt that this provided him with the opportunity to develop the essential teaching skills needed for furthering his career. In addition, he is working closely with the co-editor of *Clinical Studies in Medicinal Biochemistry*, Dr. Robert Glew, as the primary co-author of the chapter: "Gaucher's Disease, a Sphingolipidosis," a chapter that is complete and was submitted in July of 2004. He is also preparing three research articles for submission in the summer of 2004.

In the spring of 2003 and 2004, he was nominated for the University-wide "Teaching Assistant of the Year" Award. In addition, he was nominated and initiated into the Phi Kappa Phi honor society in April, 2004.

IIc. <u>DETAILED SUMMARY OF PERFORMANCE ACCOMPLISHMENTS</u> (listed per FY2002-03 reviewer's comment: "List performance accomplishments in an outline based upon the statement of work.")

Specific Aim 1: (13 tasks)

Task 1 Month 1 **Complete**

- Identification of a sample breast tumor population consisting of low, intermediate, and high hTERT mRNA expressing tumors.
- The acquisition of human breast tissue is in progress as approved by our institution's Human Research and Review Committee (HRRC)

Task 2 Month 1-6 **Complete**

- DNA/RNA has been extracted from newly selected breast tumors.

Task 3 Month 1-6 **Complete**

- Quantitative RT-PCR analysis of hTERT mRNA on newly selected breast tumors
- A figure representing hTERT mRNA levels from a random group of breast tumors is shown in Appendix A. The normalized hTERT mRNA levels are displayed as fold expression relative to the mean hTERT expression from 10 normal breast specimens (red bar), Appendix A.

Task 4 Month 12-36 **In Progress**

- Cryosectioning of breast tumors

Task 5 Month 6-12 **Complete**

- Optimize hTERT FISH assay

Task 6 Months 12-18 **Complete**

- perform hTERT FISH assay
- The attached figure displays the in situ detection of hTERT mRNA from a representative sample of breast specimens. The normalized hTERT mRNA level are also referenced and are displayed as fold expression relative to the mean hTERT expression from 10 normal breast specimens, Appendix B.

Task 7 Months 12-18 **Not Successful**

- Optimize hTERT IHC assay:
- We have determined that the current commercial antibodies to telomerase did not produce satisfactory results for immunohistochemical detection within frozen breast sections. Other researchers in the telomerase field have confirmed negative results using these same antibodies.

Task 8 Months 12-18 **Complete**

- Optimize cytokeratin-7 IHC assay

Task 9 Months 12-18 **Complete**
- Optimize vimentin IHC assay
- photo available in Appendix C.

Task 10 Months 12-18 **Complete**
- Optimize α-actin IHC assay

Task 11 Months 12-18 **Complete**
- Optimize common acute lymphocytic leukemia antigen IHC assays

Task 12 Months 18-20 **Initiated, not complete**
- Optimize IHC triple-labeling experiments

Task 13 Months 21-36 **Not Initiated**
- Perform IHC triple-labeling assay

Specific Aim 2: (5 tasks)

Task 1 Month 12-36 **In Progress**
- Creation of primary cell cultures from fresh breast tumors.
- A photo of theses cells is shown in Appendix D.

Task 2 Month 22-36 **Not initiated**
- Growth analyses of primary cell cultures

Task 3 Month 12-15 **Complete**
- Creation of GFP reporter plasmid (months 12-15)
- These constructs are displayed in Appendix E.

Task 4 Month 15-36 **Initiated**
- Optimization and flow separation of cells based on GFP levels

Task 5 Month 15-36 **Initiated**
- Characterization of hTERT-rich cells by growth rate, anchorage independence, DNA flow cytometry, telomere content.

III. REPORTABLE OUTCOMES

Publications:

Bisoffi M, Klima I, Gresko E, Durfee PN, Hines WC, Griffith JK, Studer UE, Thalmann GN. Expression profiles of androgen-independent bone metastatic prostate cancer cells indicate over-expression of the putative serine-threonine kinase GS3955. In Press in *Journal of Urology*.

Presentations: (abstracts in Appendix)

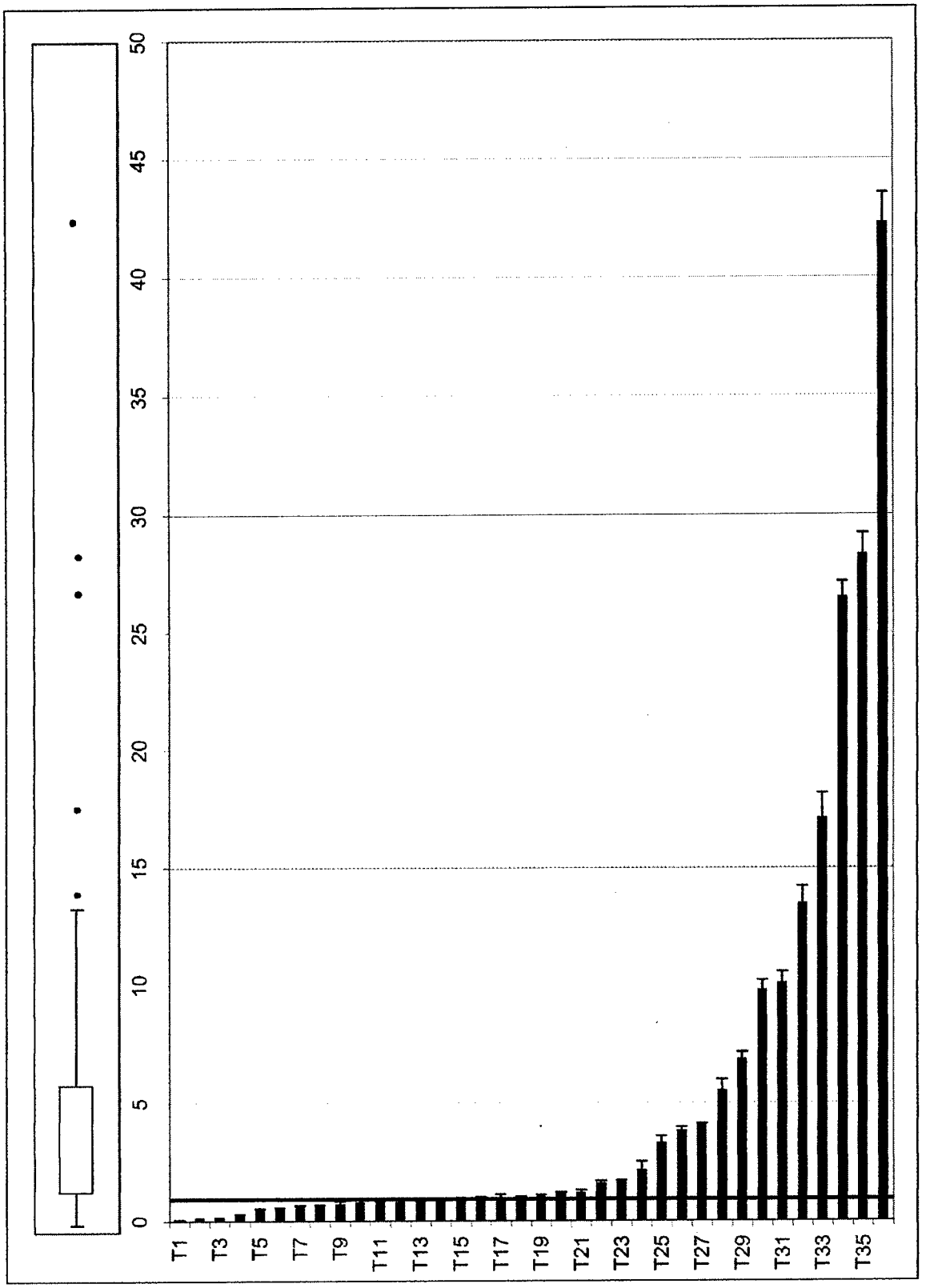
St. Jude's National Graduate Symposium, April 28-30th, 2004: "Levels of Telomere Protein mRNAs are increased in Human Breast Tumors with Decreased Telomere Content." Kimberly S. Butler, William C. Hines, Colleen A. Fordyce, and Jeffrey K. Griffith

University of New Mexico Biochemistry Research Day, April 24, 2004: "Expression of the Kinase-like protein, GS3955, in Breast Cancer." Smith JA, Hines WC, Butler KS, Griffith JK, Bisoffi M

University of New Mexico Biochemistry Research Day, April 24, 2004: "Measuring c-MYC mRNA Levels in Human Breast Cancer using Real Time PCR." Alexandra Fajardo, William C. Hines, Kimberly Butler, and Jeffrey K. Griffith.

IV. Conclusions

To date, all tasks; as outlined in the Statement of Work, are on schedule. Nearly all of the tasks contained within Specific Aim #1 have been completed, and the PI has begun preparation of a manuscript based on the results from this body of work. Regarding specific aim #2, which focuses on the identification, separation, and characterization of hTERT expressing breast tumor-derived cells, two large technical achievements have been accomplished. The PI has demonstrated the ability to grow primary cells derived from breast tumors, and has generated adenoviral particles containing the hTERT promoter/GFP reporter constructs, along with the appropriate control constructs.

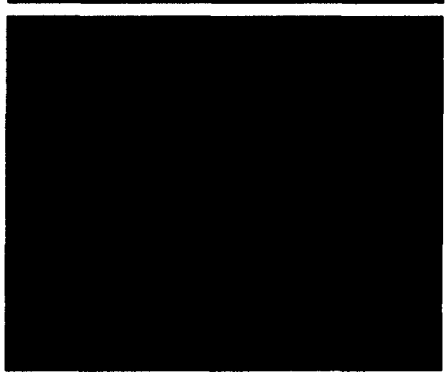


Appendix A - hTERT mRNA levels within a random group of breast tumors. The levels are expressed relative to the mean hTERT mRNA level of 10 normal breast samples (red bar). A box plot demonstrates the distribution of the hTERT mRNA levels within these tumors (left).

Histologically normal Breast
hTERT mRNA level= 1.00

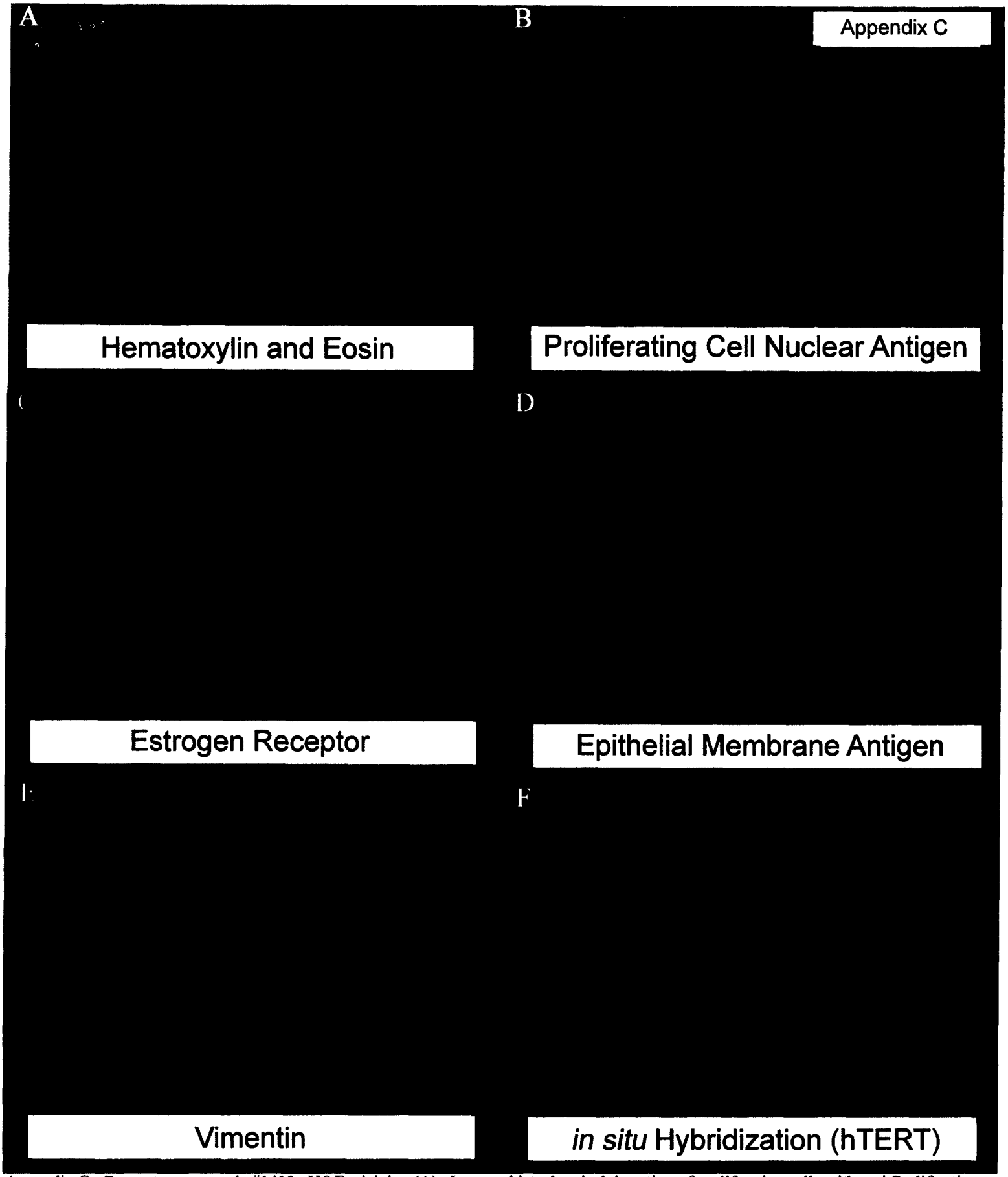
Breast tumor #189
hTERT mRNA level= 0.06

Breast tumor #342
hTERT mRNA level=0.09



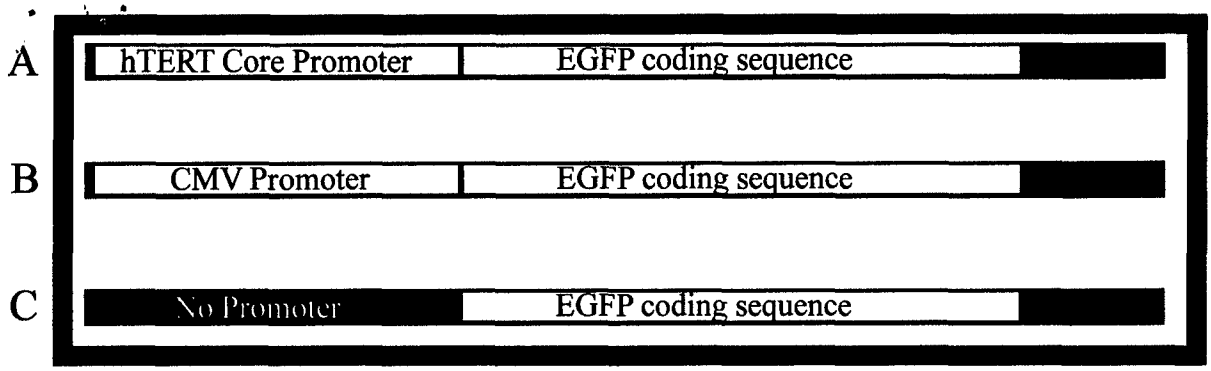
Breast tumor #866
hTERT mRNA level=10.07

Breast tumor #1257
hTERT mRNA level=17.08

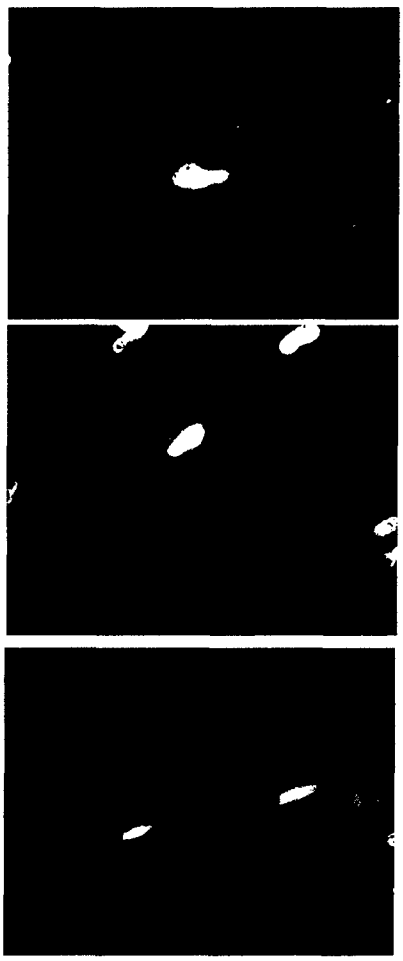


Appendix C - Breast tumor sample #1419. H&E stiaining (A). Immunohistochemical detection of proliferating cells with anti-Proliferating Cell Nuclear Antigen, (B); estrogen receptor expressing cells with anti-Estrogen Receptor, (C); epithelial cells with anti-Epithelial Membrane Antigen,(D); fibroblasts with anti-Vimentin (E). Detection of Telomerase expressing cells by in situ hybridization using a hTERT antisense probe (F).

Appendix D - Primary cell cultures derived from breast tumors. Cells before fractionization, containing epithelial cells (EC), and stromal cells (SC) are shown in panel A. The epithelial fraction (panel B), and the stromal fraction (panel C) were fractionated by differential centrifugation and placed in culture.



WI-38 fibroblasts
(do not express hTERT)



D hTERT Promoter-GFP construct
+ DSRED co-transfection

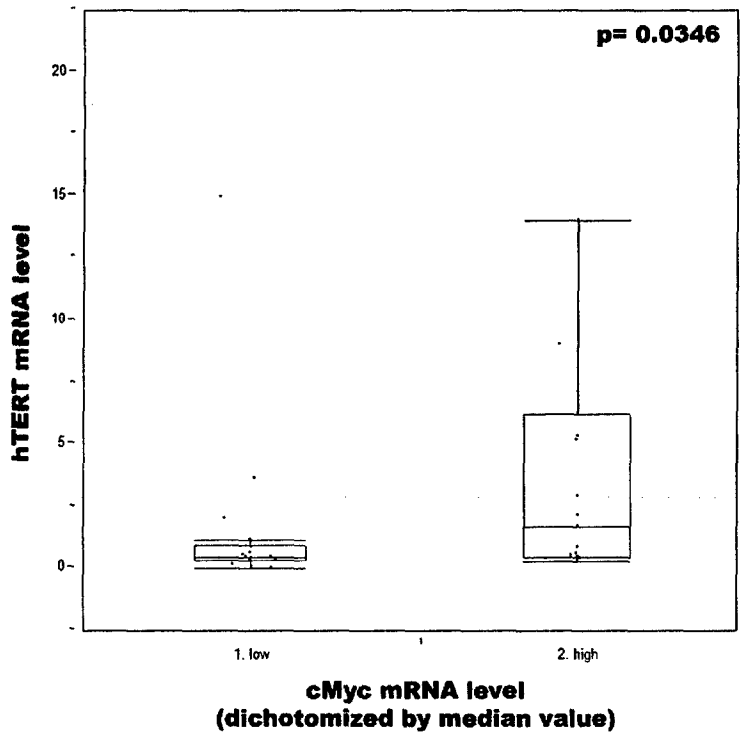
E CMV Promoter-GFP construct
+ DSRED co-transfection

F No Promoter-GFP construct
+ DSRED co-transfection

Diagragm of promoter-GFP constructs - The hTERT core promoter was amplified from human genomic DNA. The hTERT coding sequence was replaced by the GFP coding sequence (A). GFP was also placed under the control of the CMV promoter (positive control), (B), or DNA sequence with no promoter activity (negative control), (C).

These constructs were transfected into WI-38 fibroblasts, a cell line that does not contain detectable expression of hTERT mRNA or telomerse activity. They were cotransfected with DSRED plasmid, to identify transfected cells, and subsequently counterstained with DAPI These photos have been merged to show the three fluorescent channels (DAPI-Blue, GFP-Green, DSRED-Red).
As seen in panel D, the hTERT-GFP constuct does not display green fluorescence within these telomerase negative cells. The CMV promoter construct appears yellow, from the combined GFP and DSRED, (E). The Promoterless construct appears red due to the absence of GFP, (F).

These constructs were also transfected into hTERT expressing cells (photo not available). The hTERT and CMV promoter constructs demonstrated green fluorescence, whereas the promoterless construct did not display green fluorescence.



Appendix F- hTERT mRNA levels are increased in tumors dichotomized by their median cMyc mRNA level (p = 0.0346)

Appendix G

Levels of Telomere Protein mRNAs are increased in Human Breast Tumors with Decreased Telomere Content

Kimberly S. Butler BA, William C. Hines BS, Colleen A. Fordyce PhD, and Jeffrey K. Griffith PhD

Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, New Mexico

Background: Telomere length is critical to genomic stability in cancer; however, the mechanism by which telomere length is maintained is poorly understood in human tissues. To gain a better understanding of how telomere length is stabilized, telomere-associated proteins and telomere length were examined in human breast cancer samples, a tissue model of variable telomere length. Previous studies involving overexpression of telomere-associated proteins have shown that increases in telomere repeat binding factors 1 and 2 (TRF1 and TRF2), TRF1 interacting protein 2 (TIN2) and Protection of Telomeres 1 (POT1) lead to decreased telomere length. We hypothesized that telomere length is correlated with the levels of the telomere-associated proteins in human breast tumors.

Methods: 36 snap frozen human breast tumors were collected from the New Mexico Tumor Registry as approved by the University of New Mexico Health Science Center Human Research Review Committee. Anonymous patient information included age at diagnosis, tumor size, Scarff-Bloom-Richardson histopathological grade, Estrogen Receptor (ER) status, normal or abnormal DNA content (Ploidy), presence or absence of positive nodes and the fraction of cells in S-phase. Telomere length and mRNA levels of the telomere associated proteins and telomerase protein component (TERT) were assessed in breast tumor from 36 different women. Telomere length was measured as telomere content by the Telomere DNA Content Assay. Levels of the telomere-associated proteins and TERT mRNA were assessed by quantitative PCR (Figure 1).

Results: Telomere associated protein and TERT mRNAs were detected in all 36 breast tumors. The mRNA levels of TRF1, TRF2, TIN2 and POT1 had a negative linear correlation to TC ($p=0.004$, 0.006, 0.003 and 0.042 respectively; Figure 2). When the mRNA levels of the telomere-associated proteins were compared to each other, TRF1 had a positive linear correlation to TIN2 and POT1 ($p=0.009$ and 0.005; Figure 3A and 3B). Additionally, TIN2 had a positive linear correlation to POT1 ($p=0.029$; Figure 3C). In contrast, TRF2 did not correlation to any of the other telomere-associated proteins (Figure 3D). None of the telomere-associated proteins correlated with TERT (Table 1). When TRF1 was compared to clinical information no significant interactions were observed (Table 1). However, TRF2 levels were significantly increased in aneuploid tumors and showed a trend of increased expression in tumors with higher histopathological grade (Table 1). Additionally, TIN2 showed a significant increase in patients who were diagnosed at an age above 50 and POT1 showed a trend of increased

expression in larger tumors (Table 1).

Conclusion: Our study demonstrates that when the telomere-associated proteins are in high levels the telomere length is decreased in human breast tumors. The association between telomere length and telomere associated proteins has previously been demonstrated in cell lines. Our studies demonstrate a link between telomere binding proteins and telomere maintenance in human breast tumors that confirms the relationship found in cell lines. Additionally it is evident that the levels of the telomere-associated proteins that interact with TRF1 show a positive association. Future studies will examine the possibility of coordinate regulation of transcription of TRF1, TIN2 and POT1. Decreased telomere content has previously been associated with decreased survival in breast cancer, future studies will determine if telomere-associated proteins also correlate with survival in breast tumors.

Supported by Army Pre-doctoral Breast Cancer Traineeship and Breast Cancer Concept Awards.

Appendix H

Expression of the Kinase-Like Protein GS3955 in Breast Cancer

Jennifer A. Smith, William C. Hines, Kim Butler, Jeffrey K. Griffith, and Marco Bisoffi

Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM

Both, breast and prostate cancers arise from epithelial cells that have undergone genetic alterations affecting the expression and function of several protein families, including signal transduction molecules. The latter are important regulators of proliferation, differentiation, and apoptosis, physiological processes that are disturbed during cancer initiation and progression. In breast cancer, these events result in the transition of normal to hyperplastic epithelium, to carcinoma *in situ*, and ultimately to invasive and metastatic disease. We have previously shown that the novel, and as yet un-described kinase-like protein, GS3955, is up-regulated in the LNCaP/C4-2 xenograft cell line model of prostate cancer progression. We have thus hypothesized that GS3955 may be involved in tumorigenesis and/or cancer progression in general. To test this hypothesis, we have employed quantitative real-time reverse transcriptase-polymerase chain reaction (RT-PCR) to determine the expression of GS3955 in (i) 35 human breast cancer specimens of known clinical markers and pathological stage, and (ii) in 5 cancerous and matched tumor-associated normal human breast tissues. Our results indicate that GS3955 expression correlates with hTERT (telomerase) expression ($p=0.006$), and tumor size ($p=0.011$). In addition, we observed a trend between GS3955 expression and tumor grades 2 and 3 ($p=0.086$), S-Phase Fraction ($p=0.061$), and estrogen receptor (ER) status ($p=0.079$). Interestingly, GS3955 expression in normal matched tissue was consistently higher than in the corresponding tumor tissue in all 5 specimens analyzed (average=2.7±1.5-fold; range=1.3-5.2). The latter result indicates either (i) an overall loss of GS3955 during tumorigenesis, or (b) a confounding effect due to stromal cell-derived GS3955 expression. In summary, our results indicate a role of GS3955 in breast cancer tumorigenesis and progression.

Appendix I

Measuring c-MYC mRNA Levels in Human Breast Cancer using Real Time PCR

Alexandra Fajardo, William C. Hines, Kimberly Butler, and Jeffrey K. Griffith.
Department of Biochemistry and Molecular Biology, University of New Mexico School of Medicine, Albuquerque, NM

Telomeres are protein-nucleic acid structures located at the ends of eukaryotic chromosomes. They function to stabilize the chromosomal ends and thus protect the cell from acquiring genomic alterations. The DNA component is formed from a hexanucleotide repetitive element, TTAGGG, that is repeated 1,000 to 2,000 times per chromosome. Short sequences (50-100bp) of telomeric DNA is lost as a cell divides due to incomplete replication of the DNA; a mechanism known as the "End-Replication-Problem." Cells with a higher proliferative history, such as tumor cells, have dysfunctional telomeres and thus, are more likely to be genetically unstable. Telomerase, the special reverse transcriptase that lengthens telomeres, is not active in the majority of normal cells. In contrast, it is activated in most tumor cells and plays a pivotal role in cell immortalization. The catalytic subunit of telomerase, hTERT, is the known limiting factor of telomerase activity as it is not commonly expressed in the normal epithelial tissue. The proto-oncogene c-MYC correlates with cell proliferation and is over-expressed in tumors. There are known binding sites in the hTERT promoter (E-boxes) for c-MYC. Therefore, we hypothesized an association between hTERT and c-MYC mRNA expression levels. To test this hypothesis, total RNA from breast cancer tissues was reverse transcribed into cDNA using Moloney Murine Leukemia Virus (MMLV). Expression of c-MYC was measured by quantitative real time reverse transcriptase-polymerase chain reaction (QRT-PCR) using specific intron-spanning primers and a dual-labeled fluorescent probe. Plasmids carrying the c-MYC and TBP DNA sequences were used as standards. Within each sample, the expression of c-MYC was normalized to expression of the TATA-Binding Protein (TBP). Using these tools, QRT-PCRs were performed on 37 breast tumor samples with known pathology using an ABI 7000 real time thermocycler. Specifically, we assessed the potential associations between c-MYC and previously determined hTERT expression levels, expression of telomere binding proteins, telomeric DNA content, and other clinical factors including: age, node status, S-phase fraction and tumor size.